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### Sorting out cholesterol metabolism

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# CHAPTER 1

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## General introduction

**Cargo recycling**

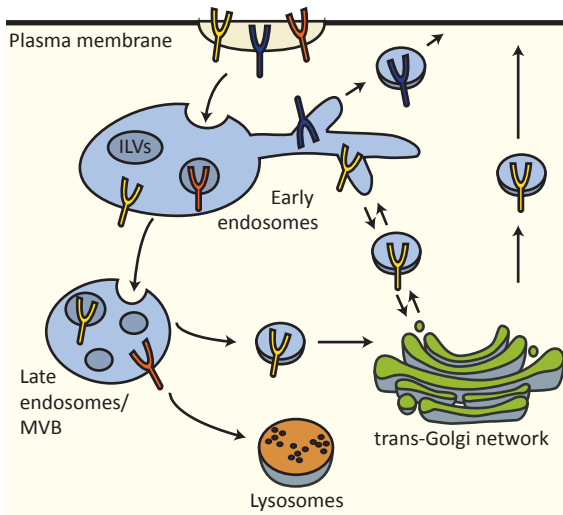
The endocytic system consists of distinct membrane compartments that act mutually to regulate the cell surface levels of integral membrane proteins and lipids (also referred to as cargos). Controlling the composition of the cell surface is essential for the regulation of various biological processes, including nutrient transport, cell signaling and cell migration, and ultimately response to intracellular and environmental changes (1).

The recycling pathway is one of the crucial endocytic trafficking steps in the regulation of cell surface levels of cargos. Recycling starts after internalization of extracellular solutes, proteins and lipids by endocytosis. Following endocytosis, the cargos are transferred to early endosomes, from whence they can be sorted to various subcellular destinations. Cargos targeted for degradation are transported to the lysosomes. Cargos not marked for degradation are retrieved from the degradative fate and subsequently recycled back to the cell surface or transported to the trans-Golgi network (TGN), a process also known as retrograde transport (1-4) (Fig. 1). Although it was previously assumed that cargo recycling occurs as an unspecific bulk process, recent studies have unveiled that specific rescue of cargos from the lysosomal fate is based on cargo sorting motifs (5, 6).

**Retromer – Retrieval of cargo from lysosomal degradation**

A central player in the process of cargo recognition at the early endosomes to prevent lysosomal degradation is retromer (7-9) (Fig. 2A). Retromer was originally identified in yeast, where it exists as a heteropentamer of five vacuolar protein sorting (Vps) proteins (10). These five Vps proteins form two subcomplexes: the cargo-selective complex consisting of Vps26, Vps29 and Vps35, and a SNX-BAR heterodimer consisting of Vps5 and Vps17, which facilitates endosomal tubule formation (10, 11). The cargo-selective trimer is evolutionary highly conserved, and its composition is essentially identical in higher eukaryotes compared to yeast (12, 13). However, in higher eukaryotes the cargo-selective trimer and the SNX-BAR dimer can independently sort various cargos (14, 15), suggesting individual roles of the two sub-complexes in higher eukaryotes (16, 17). Therefore, in higher eukaryotes retromer is defined to consist of only the cargo-selective VPS26/VPS29/VPS35 trimer, and the SNX-BAR dimer is considered to function as an independent protein complex, based on the transient interaction of these complexes.

To provide specificity to endosomal cargo sorting, retromer associates with cargo specific adaptors, such as adaptor proteins SNX27 and SNX3, which recognize specific sorting motifs. For example, SNX27 recognizes PDZ ligands, such as  $\beta$ 2 adrenergic receptor (18), and SNX3



**Figure 1. Sorting pathways of the endosomal network.** Cargos are endocytosed and sorted from the early endosomes to various subcellular destinations. Cargos targeted for degradation are sorted first into intraluminal vesicles (ILVs) and after endosomal maturation arrive in late endosomes/multivesicular bodies (MVBs). Finally, after fusion of MVBs with lysosomes the cargos are degraded. Cargos that are retrieved from the degradation pathway are either recycled back to the plasma membrane, or can be transported back to the trans-Golgi network (TGN), a process also known as retrograde transport.

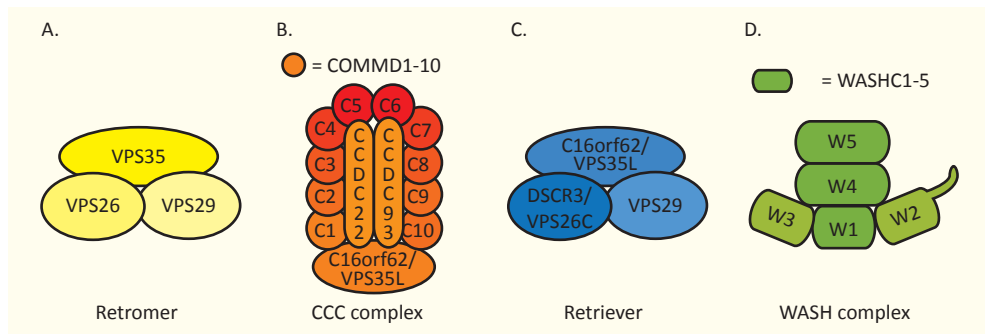
recognizes  $\emptyset$ X(L/M) motifs ( $\emptyset$  represents an aromatic residue) within transmembrane proteins, such as the cation transporter DMT1-II (19). Interestingly, adaptor proteins also affect the subcellular localization of retromer. When retromer interacts with SNX-BAR proteins, the complex resides mainly on non-branched tubules (20), whereas the SNX3-retromer complex resides on small clathrin coated vesicles (21).

Together these studies show that retromer is a promiscuous protein complex, which provides specificity to cargo recycling by associating with various adaptor proteins to facilitate trafficking of cargos with different sorting motifs to multiple subcellular destinations.

### The CCC complex – A selective cargo recognition complex

Like retromer, the CCC complex resides on early endosomes and is involved in retrieval and recycling of multiple cargos, including the low-density lipoprotein receptor (LDLR), the copper transporting P-type ATPase ATP7A, Notch and  $\alpha 5\beta 1$ -integrin (22-26). The core of the CCC complex consists of the coiled-coil domain-containing proteins CCDC22 and CCDC93 (24) (Fig. 2B). Although C16orf62 (later renamed to VPS35L) was originally considered to be a core component of the CCC complex, recent work suggests that C16orf62/VPS35L participates in retrieval, a separate heterotrimeric protein complex consisting of C16orf62/VPS35L, DSCR3 (later renamed to VPS26C) and VPS29 (27) (Fig. 2C).

The core of the CCC complex is decorated with a combination of proteins of the COMMD family; a family of proteins consisting of ten members (COMMD1-10). COMMD proteins are



**Figure 2. Composition of the protein complexes in the endosomal sorting pathway.** Composition of the multi-protein complexes: **(A)** retromer, **(B)** the CCC complex, **(C)** retriever and **(D)** the WASH complex. C1-C10 = COMMD1-10, W1-W5 = WASHC1-5.

ubiquitously expressed and are highly conserved throughout evolution (28, 29). COMMDs are characterized by the COMM domain, and are present in all vertebrates, with significant conservation among mammals and fish (28). *COMMD1*, the founder of the COMMD family, has originally been identified as the gene underlying copper toxicosis (CT) in dogs (30). CT is a hepatic copper storage disorder caused by impaired copper excretion into the bile. It has been suggested that COMMD1 regulates hepatic copper homeostasis through the interaction with the copper transporting P-type ATPase ATP7B (31). ATP7B transports excess hepatic copper into the bile, and the overall topologies and structures of ATP7B are very similar to ATP7A, and the proteins are highly homologous (33). A recent study showed that COMMD1 acts in concert with the WASH (Wiskott-Aldrich syndrome protein and scar homolog; see also paragraph 1.4) complex to facilitate the endosomal transport of the copper transporting P-type ATPase ATP7A (24, 32). Therefore, it is plausible that COMMD1 is also required for the endosomal transport of ATP7B and, consequently, in impaired ATP7B-mediated biliary copper excretion (30, 32).

COMMDs are vital for embryonic development in mice, as depletion of *Commd1*, *Commd6*, *Commd9* or *Commd10* results in embryonic lethality (23, 34-36). Interestingly, these embryos die at different stages of gestational development, suggesting that the COMMD proteins control specific biological processes. This hypothesis is strengthened by multiple studies. Firstly, although all ten COMMD proteins can interact with each other through their COMM domain (28, 37), specific COMMD combinations are preferential; for instance COMMD10 binds preferably to COMMD2 and COMMD5, whereas COMMD9 only weakly binds to COMMD6 (38). Secondly, endosomal sorting of multiple cargos is only dependent on specific

COMMD proteins; not all COMMDs can bind to ATP7B, and proper Notch levels at the cell surface rely only on the expression of COMMD5 and COMMD9 (23). Altogether, these studies suggest that the CCC complex is a transitive protein complex in receptor sorting, with a composition that is likely circumstance and cargo specific.

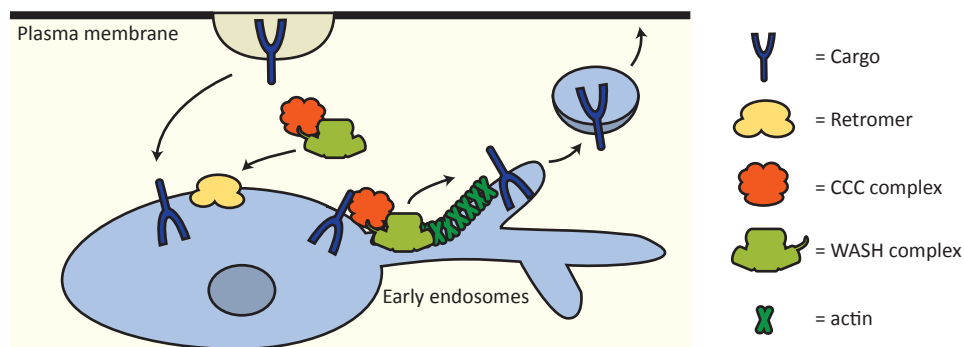
### **WASH – Targeting of endosomal cargo**

After endocytosis, endosomal cargo transport is further facilitated by the WASH complex (39). WASH is a pentameric protein complex, consisting of components WASHC1-5 (40-45) (Fig. 2D). In mouse embryonic fibroblasts (MEFs) and *Drosophila*, depletion of individual WASH components results in downregulation of all WASH proteins, suggesting that the WASH components function interdependently to maintain the integrity and the function of the complex (46, 47).

WASH is recruited to the endosomes via an interaction between the “tail” domain of WASH component WASHC2 and retromer component VPS35 (26, 48). In turn, WASH recruits the CCC complex via an interaction between CCDC22/93 and WASHC2 (24, 26). Next to VPS35-dependent endosomal recruitment of WASH, a recent study proposed that the endosomal localization of the WASH complex can be VPS35-independent (27), possibly due to a direct interaction between the WASH complex and negatively charged endosomal lipids (27, 40).

WASH provides morphological stability to endosomal and lysosomal structures (40, 44, 45, 47). In line, endosomes of WASH-deficient MEFs are collapsed and exhibit various atypical morphologies, including clusters, vacuolar-like structures, and short actin-free sorting tubules (47). Depletion of WASH in MEFs also results in collapsed and aggregated lysosomes (47), and both endosomes and lysosomes are localized to the perinuclear region WASH-deficient MEFs (47).

Along with its role in overall endo-lysosomal organization, WASH contributes to the segregation of endosomal retrieval and degradative domains. WASH acts a nucleation-promoting factor (NPF) by recruiting and activating the actin-related protein 2/3 (Arp2/3) complex (39-41, 44, 45, 49-51). Arp2/3 nucleates branched actin on the endosomal membrane, forming sorting domains. These domains restrict the lateral movement of cargos on the endosomes and facilitate sorting of cargos away from the degradative pathway (52). Depletion of WASH results in impaired endosomal cargo recycling leading to increased lysosomal degradation of cargos (22, 47). Furthermore, these endosomal actin patches induce membrane curvature



**Figure 3. CCC- and WASH-mediated endosomal cargo sorting.** After endocytosis, cargo is sorted at the endosomes. Recognition of cargo by retromer prevents trafficking of the cargo to the lysosomes for degradation. The WASH complex is recruited to the endosome through an interaction of the WASH component WASHC2 with the retromer subunit VPS35, subsequently, the WASH complex recruits the CCC complex via the binding of WASHC2 to CCDC22/93. The WASH complex facilitates deposition of branched actin patches that form restricted endosomal domains and induce membrane curvature, which after fission form vesicles to transport cargos back to the plasma membrane.

and stabilize tubular micro-domains, which after fission form vesicles to transport cargos to their final subcellular destination (Fig. 3) (40).

Altogether, *in vitro* studies have shown that WASH is a crucial protein complex in facilitating the transport of a plethora of membrane proteins to multiple subcellular destinations, including retrograde endosome-to-Golgi transport of CI-MPR (45) and endosome-to-cell surface transport of ATP7A (24), but the contribution of the WASH complex to physiological processes remains unclear.

### Diseases related to defects in the endosomal sorting machinery

As the endosomal sorting machinery is involved in proper functioning of various cellular processes (1), disturbance of this machinery can result in a variety of clinical syndromes (Table 1). Mutations in retromer are at the basis of multiple diseases, including gastric and colorectal cancers (53), as well as multiple neurodegenerative disorders, including Alzheimer's and Parkinson's diseases (54). The first indication that retromer was involved in Alzheimer's disease was provided when patients with Alzheimer's disease were found to have reduced levels of VPS26 and VPS35 in affected regions of the brain (55). Later studies indicated that depletion of VPS35 enhances amyloid  $\beta$  (A $\beta$ ) production, the pathogenic fragment in Alzheimer's disease (56).

Multiple *VPS35* mutations have been linked to Parkinson's disease, although it is not clear whether all variants are causative of the disease (57). The most prevalent causal variant is *VPS35* p.D620N (57). Although this variant is located within the *VPS35*-*VPS29* binding site, it does not affect retromer trimer formation or folding (58). Rather, WASH recruitment to endosomes is decreased due to reduced affinity of the *VPS35* D620N variant with the WASH component *WASHC2* (58, 59), ultimately resulting in hindered cargo trafficking to the Golgi, plasma membrane and mitochondria. The D620N mutation results in impaired degradation of the Parkinson's disease related protein  $\alpha$ -synuclein by preventing both autophagosome formation (58) and retrograde trafficking of mannose-6-phosphate receptor (CI-M6PR) (60). Furthermore, incorrect trafficking of multiple mitochondrial addressed cargos due to the *VPS35* D620N mutation results in impaired mitochondrial fusion and increased mitochondrial sensitivity to mitochondrial stressors (61), both of which are pathogenic hallmarks of Parkinson's disease (62). Interestingly, the *VPS35* variant P316S has also been associated with familial Parkinson's disease (63), but characterization of this mutation in HeLa cells showed no distinct effect on retromer trimer formation or receptor sorting (64); this could imply that if the P316S variant is indeed the causative mutation in Parkinson's disease, this is due to an as of yet unidentified function of retromer.

Mutations in WASH components have been associated with developmental and neurological diseases. Heterozygous mutations in *WASHC5* have been linked to autosomal dominant hereditary spastic paraplegia (HSP) (65, 66). Although these mutations do not affect the formation of the WASH complex or its endosomal localization, characterization of the HSP causing *WASHC5* p.N471D mutation showed that this variant decreased the protein levels of the WASH components *WASHC1* and *WASHC2*, as well as disturbed endo-lysosomal structure and defects in cell growth, phagocytosis and lysosomal function (67). *WASHC4* mutations have been identified in patients with non-syndromic autosomal recessive intellectual disability (68), while homozygous mutations in *WASHC5* have been reported in patients with Ritscher-Schinzel/3C syndrome (RSS) (69).

The biological importance of the CCC complex is reflected in X-linked intellectual disability patients, who suffer from a severe developmental disorder caused by hypomorphic mutations in *CCDC22*, resulting in decreased expression of the whole CCC complex (70, 71). Beyond developmental and neurological defects, XLID patients also suffer from increased serum copper and ceruloplasmin levels (24), likely due to impaired endosomal trafficking of ATP7B. Our recent work suggests that mutations in *CCDC22* lead to impaired recycling of the low-density lipoprotein receptor (LDLR) (22), the key receptor for the clearance of



Table 1. Summary of pathogenic mutations of the endosomal sorting machinery

Protein complex	Gene	Mutation	Condition	Phenotype	Plasma lipids	Source
CCC complex	CCDC22	p.Thr17Ala	Ritscher Schinzel syndrome 2	X-linked intellectual disability,	Total-C↑	70, 71
		p.Tyr57Cys		Posterior fossa defects, Cardiac malformations, Minor abnormalities of the face and distal extremities	LDL-C↑ HDL-C↑	
WASH complex	WASHC4	p.Pro1019Arg	Autosomal recessive intellectual disability	Intellectual disability, Severely delayed motor development, Short stature	?	68
WASH complex	WASHC5	c.3335+2T>A c.3335+4C>A c.3335+8A>G	Ritscher–Schinzel/3C syndrome	Intellectual disability Craniofacial abnormalities Variable: Dandy Walker malformation Variable: atrial and ventricular septal defects	Total-C↑ LDL-C↑ HDL-C↑	69
WASH complex	WASHC5	p.Arg171Ter p.Arg228Ter p.Tyr235Terfs p.Asn471Asp	p.Leu619Phe p.Val626Phe p.Gly696Ala p.Leu1009Phefs	Spasticity, Dandy-Walker like malformation, Atrioventricular septal defects	?	65, 66
Retromer	VPS35	p.Pro316Ser p.Tyr507Phe p.Arg524Trp p.Ile560Thr p.Met571Ile	p.His599Arg p.Met607Val p.Asp620Asn p.Leu774Met p.Glu787Lys	Tremor, Rigidity, Bradykinesia, Postural instability	?	57

plasma LDL cholesterol (LDL-C) (72). This perturbed LDLR recycling likely causes the hypercholesterolemic phenotype that has been observed in XLID patients (22). In an RSS patient carrying homozygous mutation in *WASHC5*, high plasma LDL-C levels were also seen (22). Together, the latter data suggest that the CCC and WASH complexes are not only involved in developmental and neurological processes but are also essential to maintain cholesterol homeostasis.

Currently no mutations in *COMMD* genes have been found in humans, which may suggest that detrimental mutations in *COMMD* genes lead to premature death, as has been seen in mice. Interestingly, in contrast to mice, dogs deficient in *COMMD1* do not die during embryogenesis, a fact which might suggest that loss of *COMMD1* can be compensated by other *COMMD* proteins during embryonic development in dogs.

### **Aims and scopes of this thesis**

It has been well established that the endosomal recycling pathway is crucial for plasma membrane expression of a plethora of cargos (73, 74). Recently, we established that the endosomal cargo LDLR is highly dependent on endosomal sorting complexes CCC and WASH (22, 75). LDLR is the key receptor for cellular uptake of cholesterol, which is indispensable for the growth and viability of mammalian cells and a precursor of steroid hormones, bile salts, and several vitamins. However, excess LDL-C is a major risk factor for atherosclerotic cardiovascular disease (ASCVD) (76). ASCVD and its clinical manifestations, such as ischemic heart disease and stroke, are worldwide the leading cause of morbidity and mortality (77). Therefore, all proteins involved in the regulation of receptor-mediated cholesterol clearance might be potential therapeutic targets to combat ASCVD. In this thesis we aim to further elucidate the molecular regulation of the endosomal sorting machinery in LDLR recycling, and investigate the contribution of this machinery to cholesterol homeostasis and atherosclerosis. We focus specifically on the protein complexes CCC, WASH, retromer and retriever.

**Chapter 2** provides an overview of the LDLR life cycle by giving a mechanistic overview of the regulation of LDLR trafficking (i.e. endocytosis, recycling and degradation). This overview highlights the current knowledge gap in the molecular regulation of the intracellular LDLR trafficking pathway, and we suggest that filling this gap may help to explain unresolved cases of hypercholesterolemia, as approximately 40% of hypercholesterolemia patients have no mutations in the known hypercholesterolemia genes, such as *LDLR*, *APOB*, *PCSK9* and *ARH* (*LDLRAP1*) (78)

In **chapter 3** we review novel insights into the molecular regulation of LRP1 and LDLR in cholesterol homeostasis, at both cellular and organismal levels. We discuss the pleiotropic role of LRP1 and describe the current knowledge of LRP1-mediated processes: **1)** clearance of ApoE-rich chylomicron remnants, **2)** efflux of cholesterol to HDL via ABCA1, and **3)** insulin-induced Glut2 translocation to the plasma membrane. We describe new insights in endosomal protein complexes involved in LDLR endocytosis, and the compensatory role of LDLR in LRP1 function.

**Chapter 4** describes our investigation of the contribution of the hepatic CCC complex to the control of plasma cholesterol levels and atherosclerosis. Our study showed that in addition to recycling of LDLR (22), the recycling of LRP1 also relies on the CCC complex. We furthermore demonstrated that hepatic depletion of the CCC complex in mice with a human-like lipoprotein profile accelerates atherogenesis, and assessed the role of other COMMD proteins in CCC-mediated LDLR trafficking. Although it has been thought that COMMD proteins have unique functions, we showed that hepatic depletion of the CCC components *Commd1*, *Commd6*, *Commd9* or *Ccdc22* all result in a comparable increase in plasma cholesterol levels. For the first time, we uncovered that hepatic expression of all COMMD proteins and CCC core components (CCDC22, CCDC93, C16orf62/VPS35L) rely on each other, as loss of any of these CCC subunits results in destabilization of the CCC complex. In summary, in this chapter we revealed that all members of the COMMD family are required to maintain normal plasma cholesterol levels by facilitating endosomal transport of LDLR and LRP1 back to the plasma membrane.

In **chapter 5** we investigated the roles of the WASH complex, retromer and retriever in LDL and HDL metabolism. Our *in vitro* studies indicated that the WASH complex mediates recycling of LDLR (22). Here, we provided *in vivo* evidence that the hepatic WASH complex is involved in the hepatic uptake of LDL-C by orchestrating the endosomal recycling of LDLR and LRP1. Scavenger receptor class B type 1 (SR-BI) is the main receptor for hepatic uptake of HDL-CE, and we found that hepatic loss of WASH reduces hepatocyte surface levels of SR-BI. Kinetic studies showed that hepatic uptake of cholesterol esters from HDL is also reduced by the loss of the WASH complex, likely due to impaired endosomal trafficking of SR-BI to the plasma membrane. In addition, we provided genetic evidence that the CCC and WASH complexes act together to facilitate endosomal trafficking of the lipoprotein receptors LDLR, LRP1 and SR-BI. Interestingly, although recent *in vitro* studies have shown that retromer is required for the endosomal localization of WASH (26, 48), our *in vivo* data suggest that WASH-mediated lipoprotein receptor recycling can be both retromer-dependent (e.g. for

LDLR and LRP1) and -independent (e.g. for SR-BI). In addition, our results suggest that hepatic retromer regulates triglyceride metabolism in a WASH and CCC-independent manner. Lastly, we found that hepatic ablation of the retriever component *Dscr3/Vps26c* leads only to an increase in plasma LDL-C, but not HDL-C levels, suggesting that like retromer, retriever only affects LDLR and LRP1 but not SR-BI recycling.

In **chapter 6**, we investigated whether the altered morphology of the endo-lysosomal network in liver specific *Washc1* knockout mice affects hepatic cholesterol and bile acid metabolism. We hypothesized that perturbed endo-lysosomal architecture and localization would hinder intracellular cholesterol transport, resulting in perturbed cholesterol sensing and, ultimately, cholesterol and bile acid metabolism. Interestingly, even though the expression of several genes of the cholesterol and bile acid metabolism pathways were affected upon hepatic WASH depletion, no effects were present on whole body cholesterol synthesis, cholesterol excretion, or bile acid metabolism. Overall this study suggests that under the studied conditions the alteration in endo-lysosomal architecture affects neither hepatic cholesterol homeostasis nor bile acid metabolism.

Finally, **chapter 7** provides a critical discussion of the major findings of this thesis. We place our novel findings in the context of the current understanding of the endosomal sorting machinery and its role in cholesterol homeostasis, and discuss the possible clinical impact of these findings.

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